Introduction to Flow Cytometry

presented by:

Flow Cytometry Core Facility

Biomedical Instrumentation Center

Uniformed Services University

Topics Covered in this Lecture

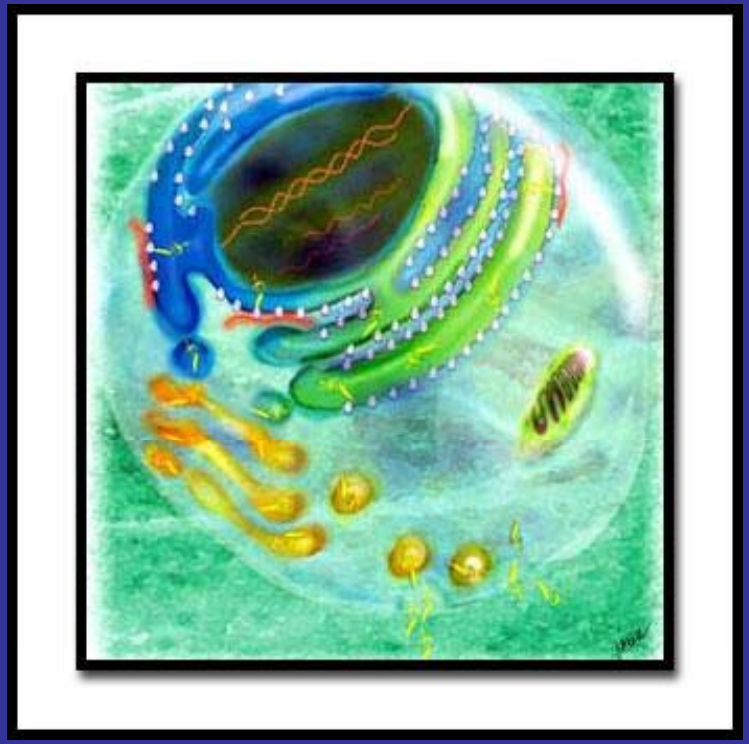
- What is flow cytometry?
- Flow cytometer instrumentation.
- The use of fluorochromes in flow cytometry.
- Immunophenotyping.
- Compensation.
- Data analysis and gating.
- Clinical applications.
- Research applications.

Flow Cytometry-- a.k.a. FACS

- Flow cytometry is a technique used to measure the physical and chemical properties of cells or cellular components.
- Cells are measured individually, but in large numbers.
- Synonymous with FACS (fluorescence-activated cell sorter).
- Also, simply referred to as “Flow.”

“Seeing” Cells

- Microscopists visualize cells based on their morphology and staining characteristics.
- Flow cytometrists measure cells based on similar characteristics.
- Hence, using flow cytometry, a cell can be “seen” both qualitatively and quantitatively.



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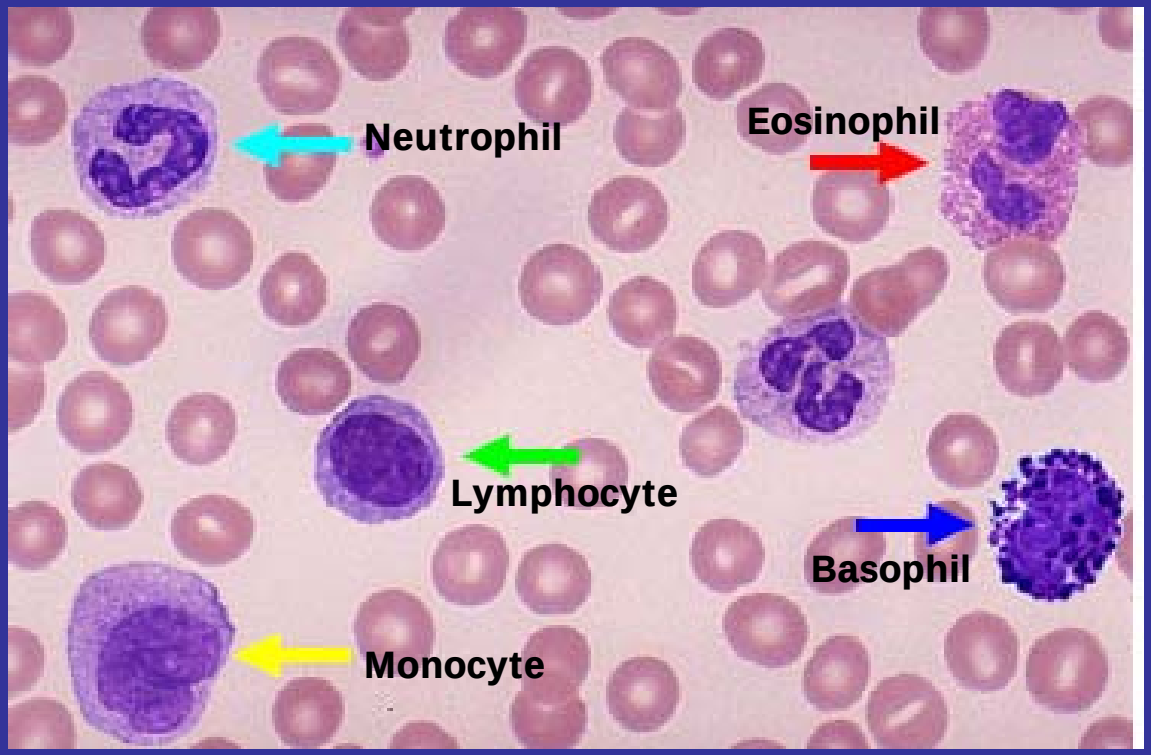
Historically...

- In the clinical lab, mixed cell populations of the blood were evaluated manually by microscope.
- In the 1950's, the Coulter counter automated cell counting based on size.



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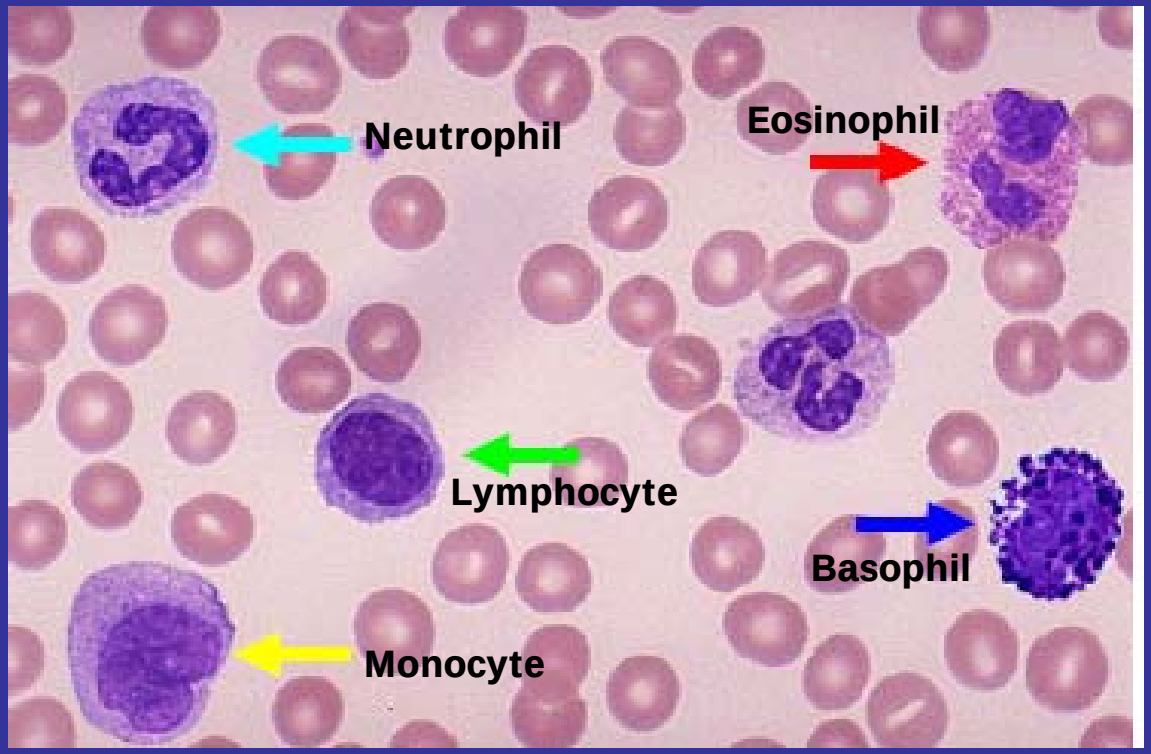
- By the 1970's, a method was needed to automatically separate living cells into subpopulations for further study.

Historically...

- In 1960, Dr. Louis Kamentsky, in collaboration with IBM, developed an automated optical scanner that scanned cell preparations on slides.
- Inferior optical and computer techniques at that time led him to develop a fixed scanner that detected cells, passing in single file, based on their light scatter and absorption.
- In 1974, Dr. Leonard Herzenberg of Stanford patented a device that sorted living cells into collection vessels for further use in biological analyses – the first FACS.



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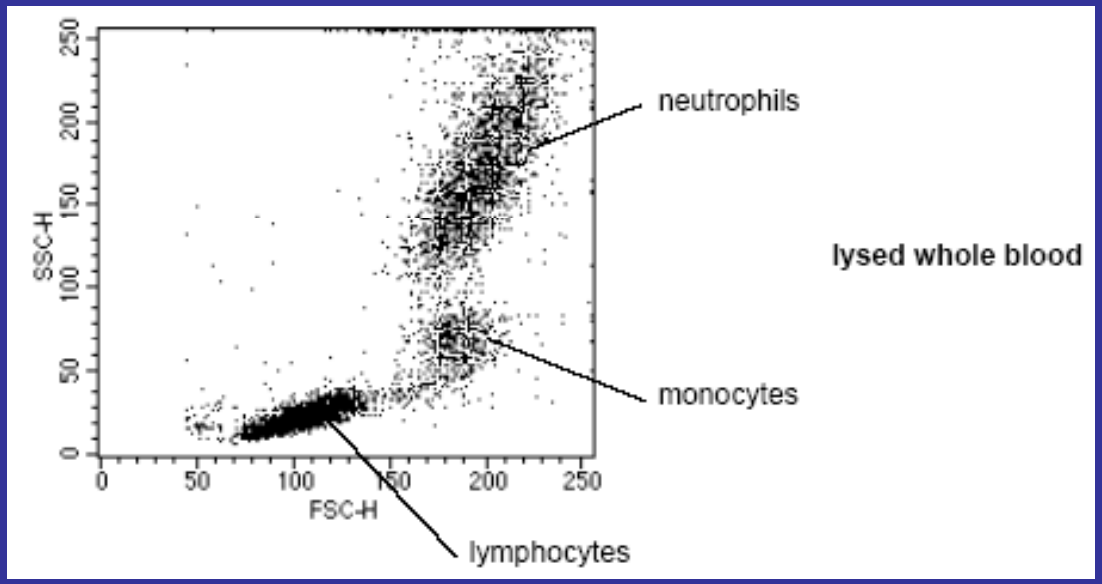
Then...

...and Now.

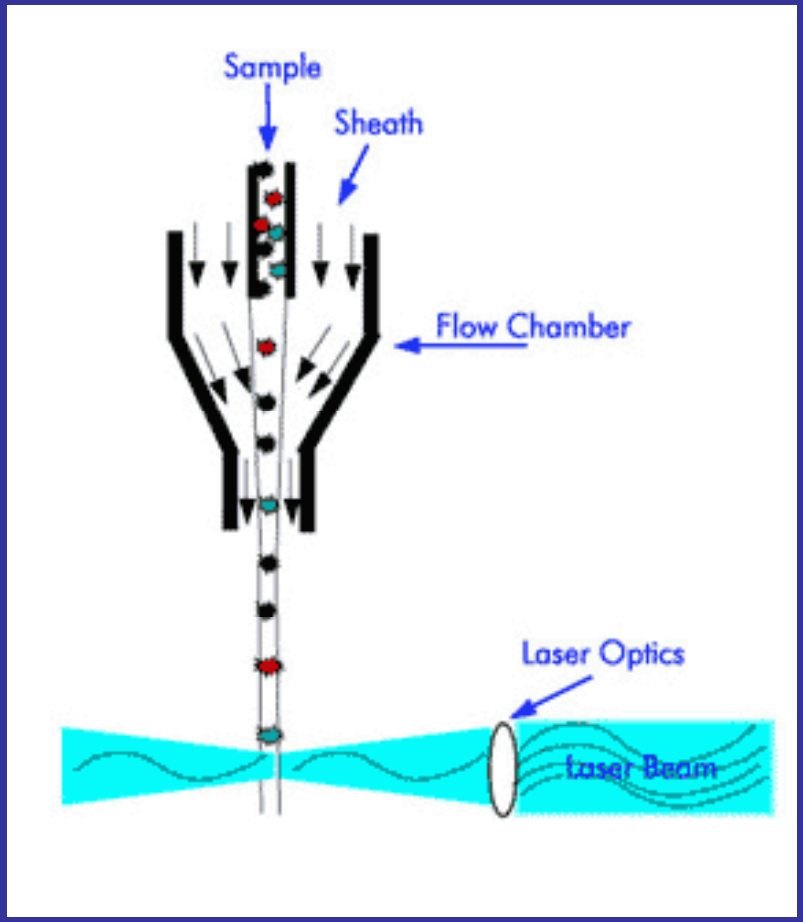


Introduction to Flow Cytometry: A Learning Guide; Becton Dickinson

Flow Cytometer Instrumentation

- There are four general components of a flow cytometer:
  - Fluidics
  - Optics
  - Detectors
  - Electronics
- Understanding how a flow cytometer operates is critical to the design and execution of flow cytometry experiments.

Flow Cytometer Fluidics

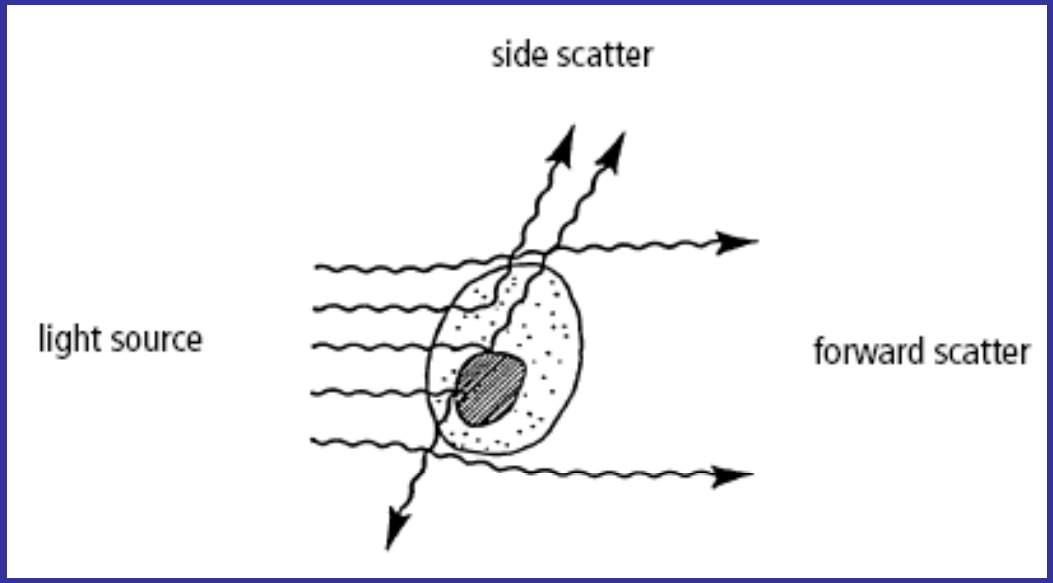


www.biology.berkeley.edu

- The cell sample is injected into a stream of sheath fluid.
- By the laminar flow principle, the sample remains in the center of the sheath fluid.
- The cells in the sample are accelerated and individually pass through a laser beam for interrogation.

Light Scatter

- When a cell passes through the laser beam, it deflects incident light.
- Forward-scattered light (FSC) is proportional to the surface area or size of a cell.
- Side-scattered light (SSC) is proportional to the granularity or internal complexity of a cell.



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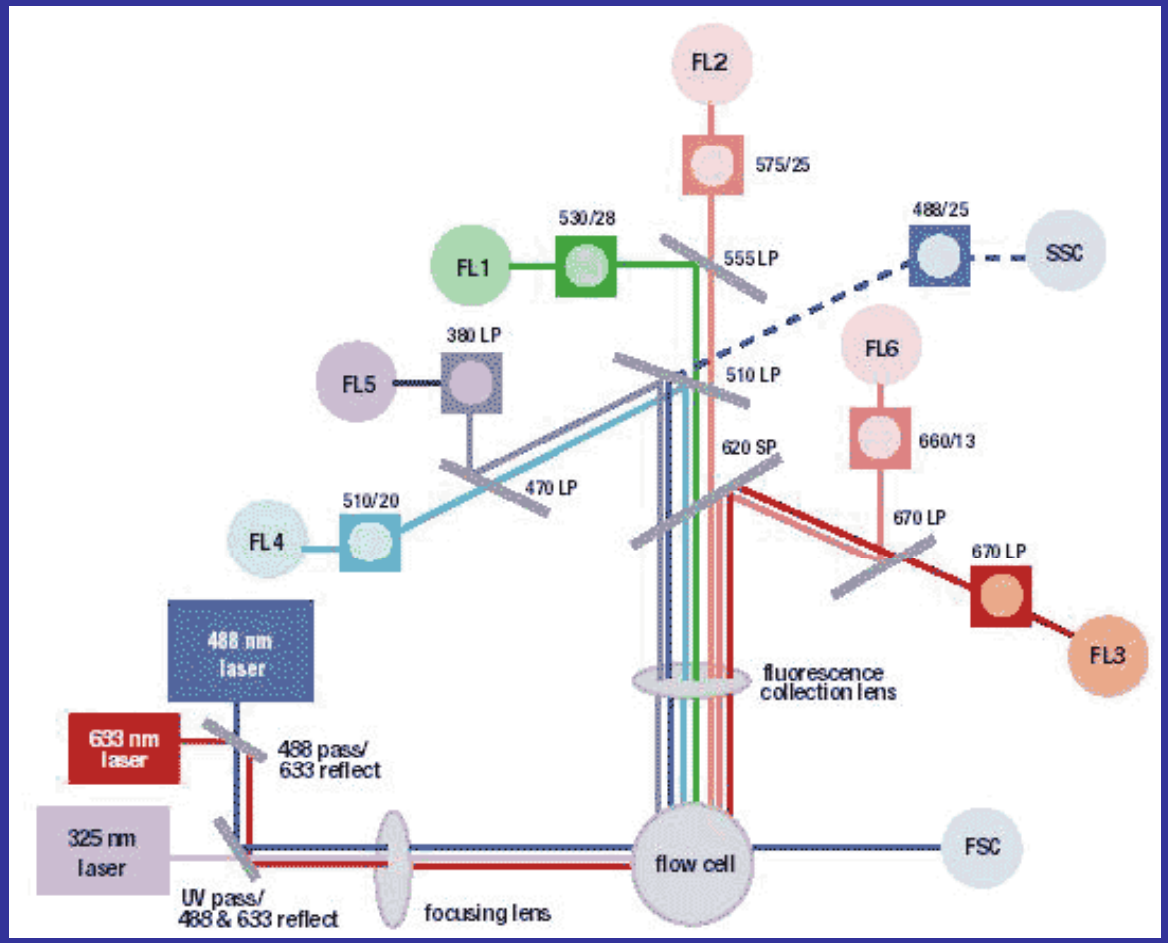
Flow Cytometer Optics

- Light emitted from the interaction between the cell particle and the laser beam is collected by a lens.
- The light moves through a system of optical mirrors and filters.
- Specified wavelengths are then routed to optical detectors.

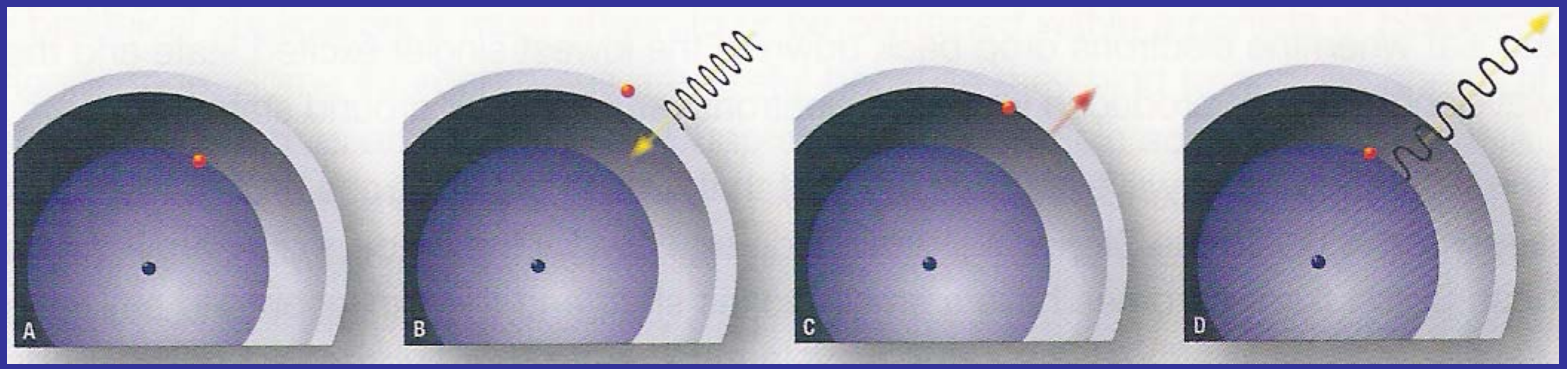


Fluorescence

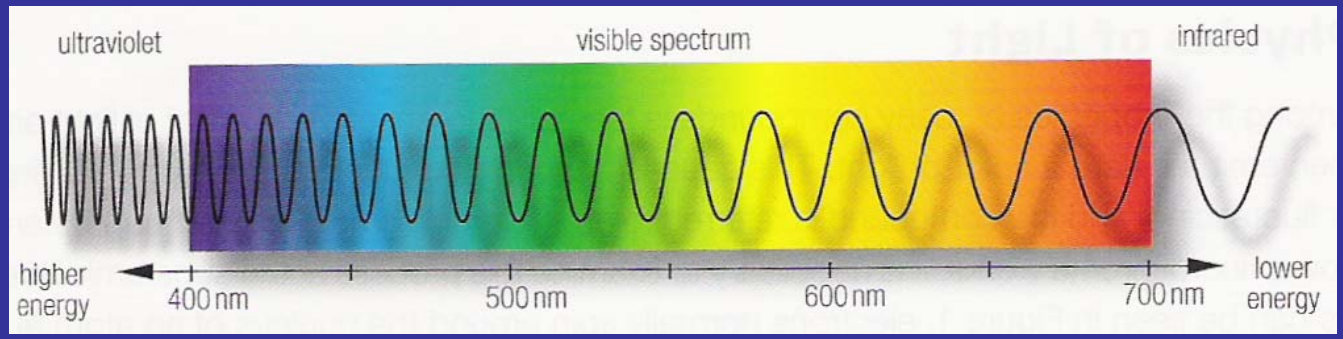
- In modern flow cytometers, more than one laser is focused on the sample stream.
- In this way, not only can cells be measured based on their size and internal complexity, but they can also be measured based on their fluorescent signal intensity.
- Fluorescence is typically “bestowed” upon a cell through the use of fluorescent dyes called fluorochromes.

Physics of Light

- Photons of light excite electrons to a higher energy state, which then release energy as heat and light.



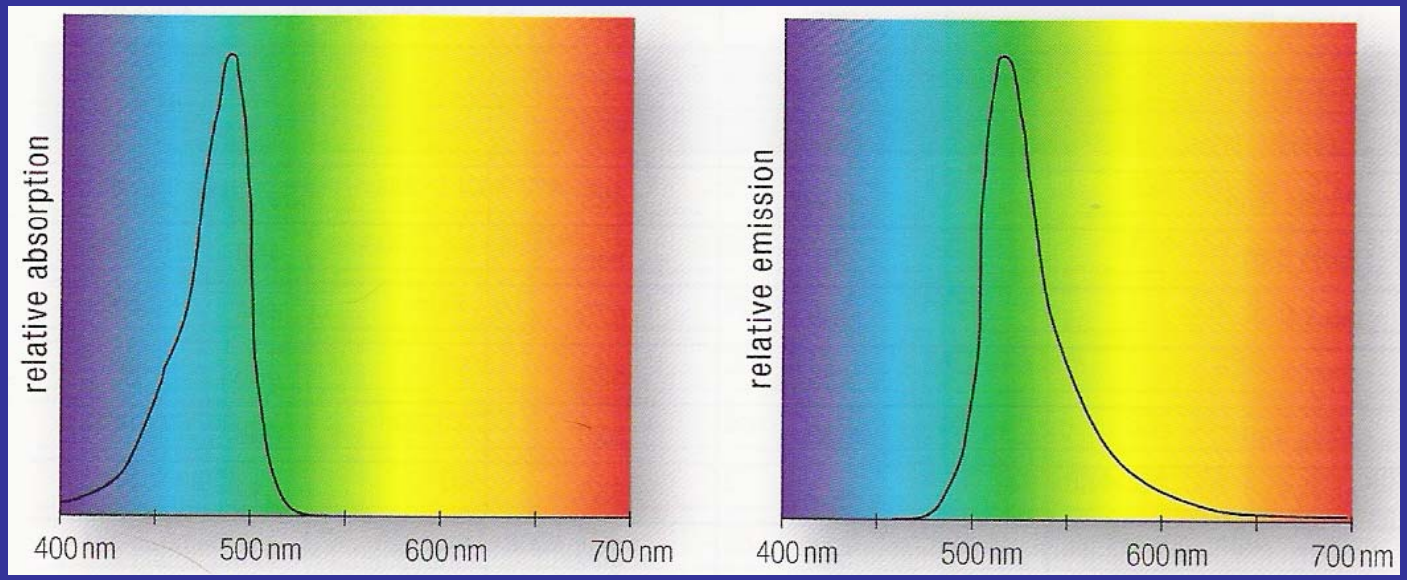
- Each type of fluorochrome exhibits its own Stokes shift in this regard and emits light of a specific wavelength.



Guide to Flow Cytometry; DakoCytomation

Fluorochrome Emission

- The laser beam excites the fluorochrome at a specific wavelength (absorption) and the fluorochrome emits light at a separate wavelength (emission).
- Note that absorption color differs from emission color.



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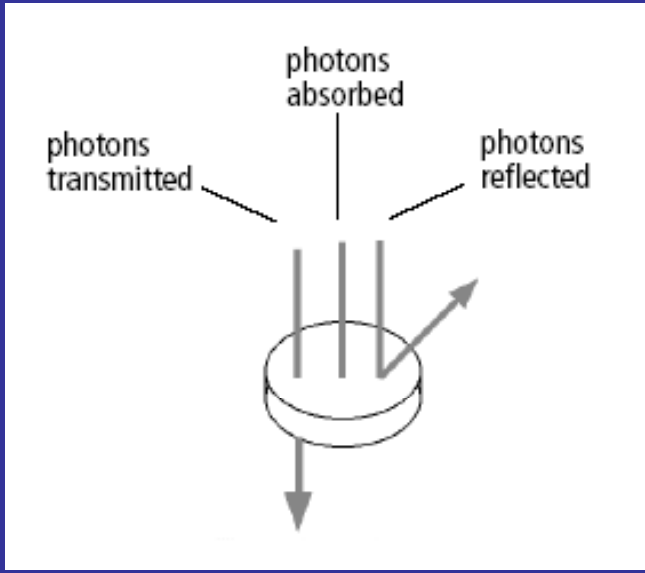
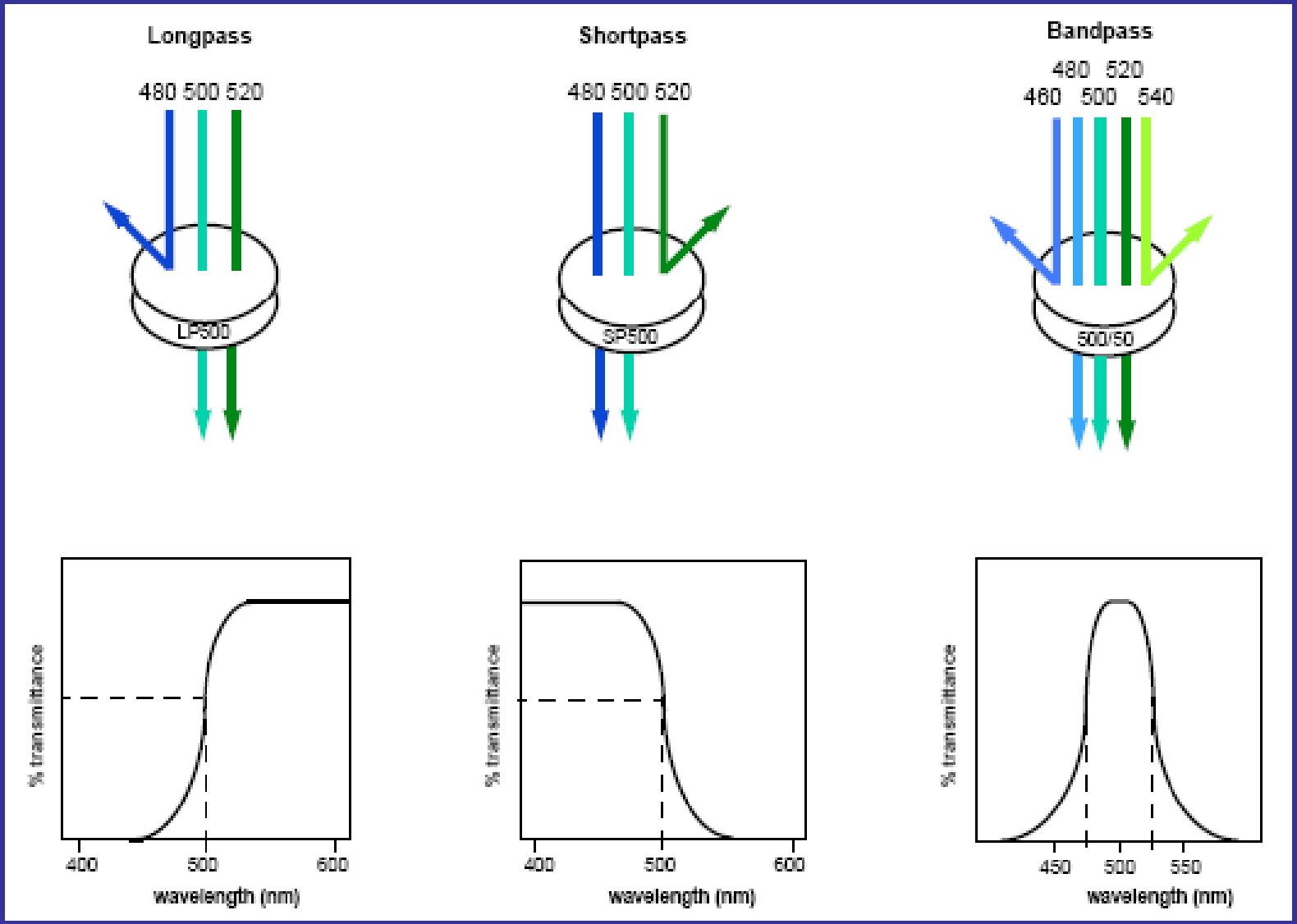
Flow Cytometer Optics

- The emission wavelength of a fluorochrome can be optically separated from other confounding light through the use of optical filters.



- Shortpass, longpass, and bandpass optical filters are used to limit each fluorochrome emission to a desired wavelength.

Flow Cytometer Optics



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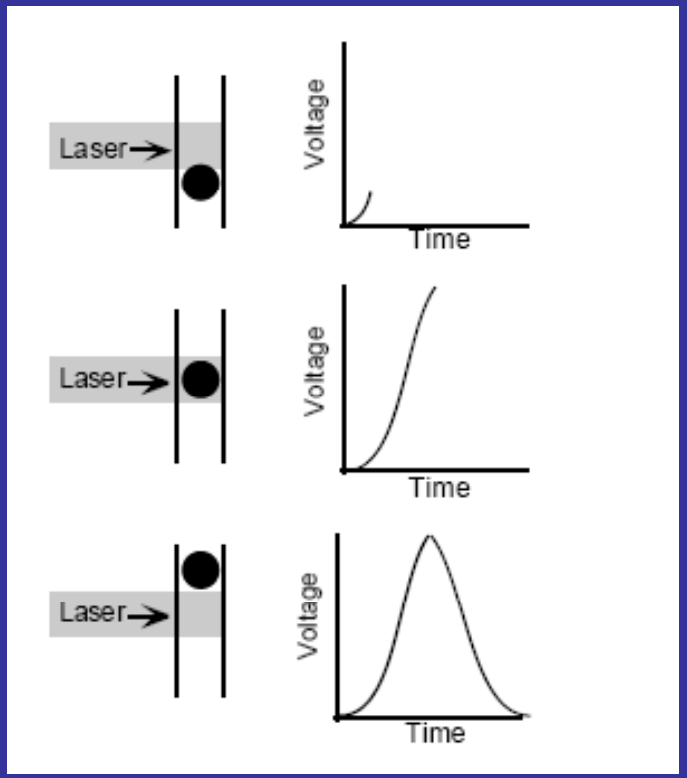
Flow Cytometer Signal Detection

- As a particle passes through the laser and fluoresces, it is detected by a photodetector (PMT).
- An electrical pulse (the voltage pulse) is generated and is processed by the signal processing electronics of the flow cytometer.



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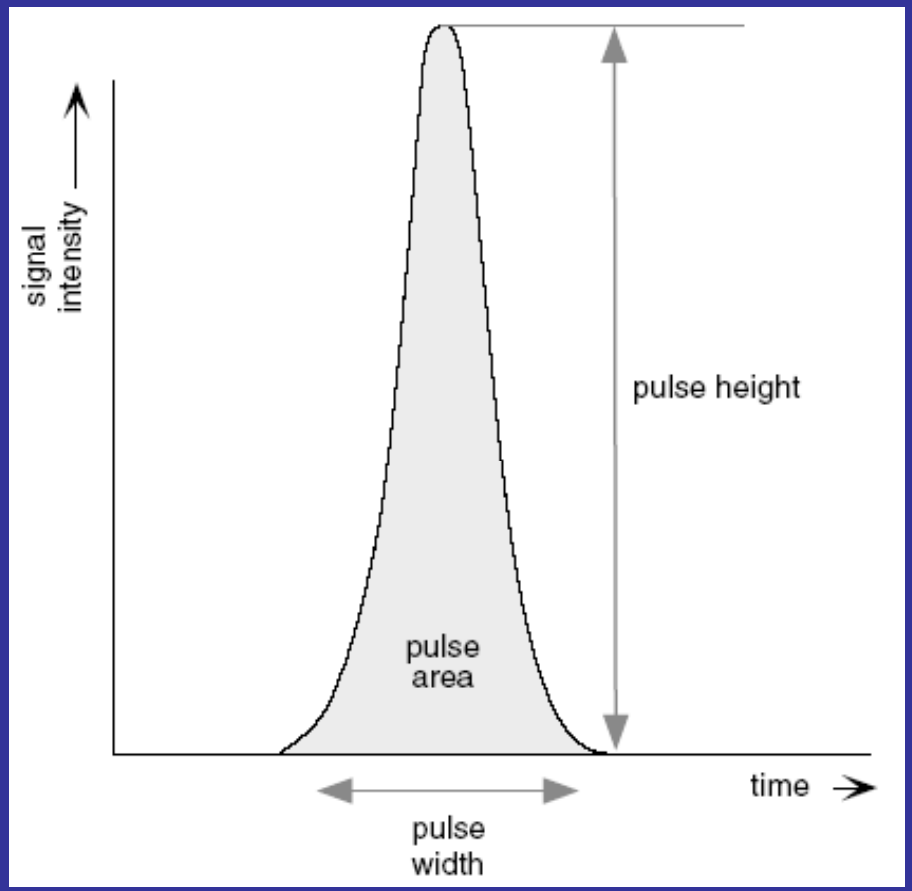
Flow Cytometer Electronics



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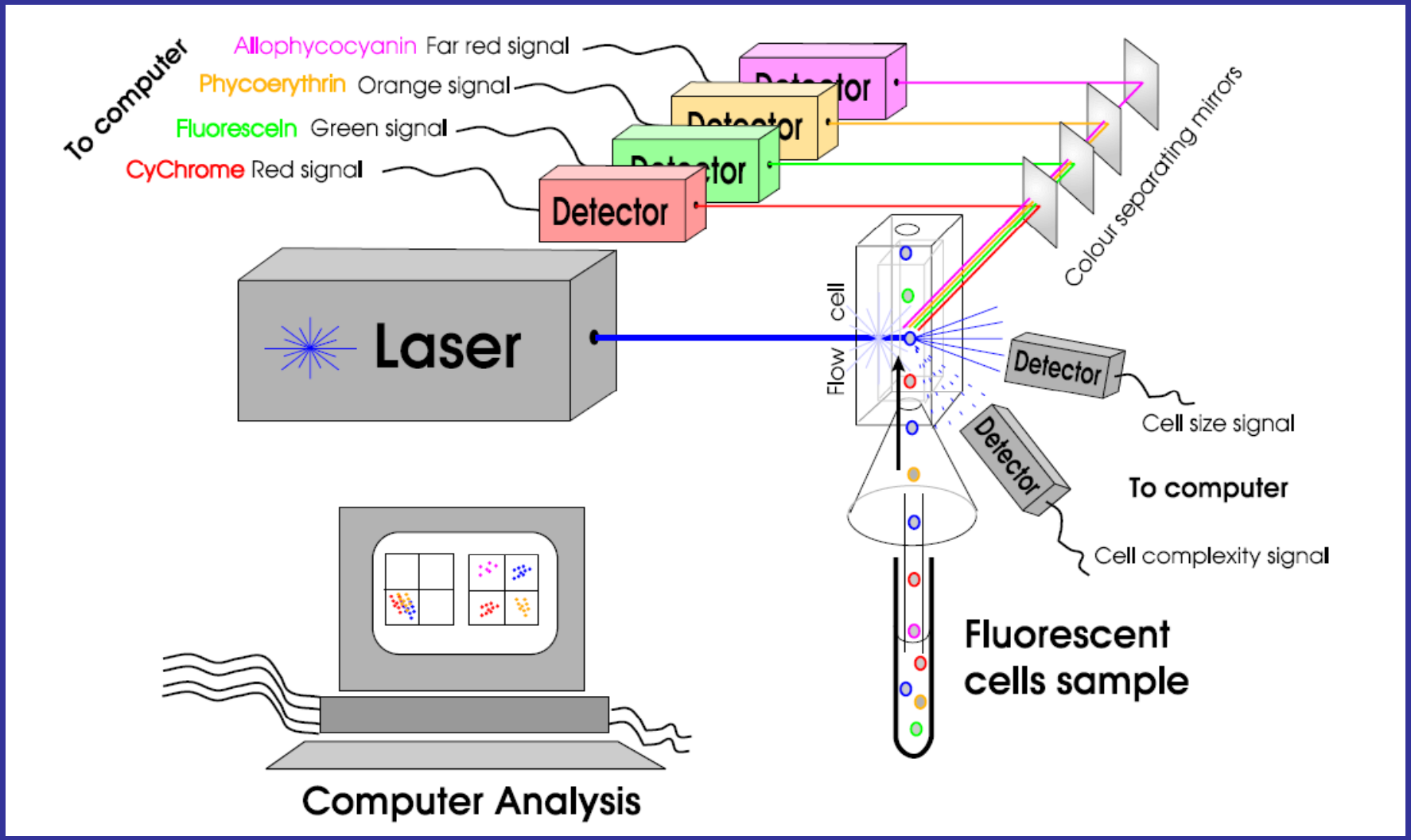
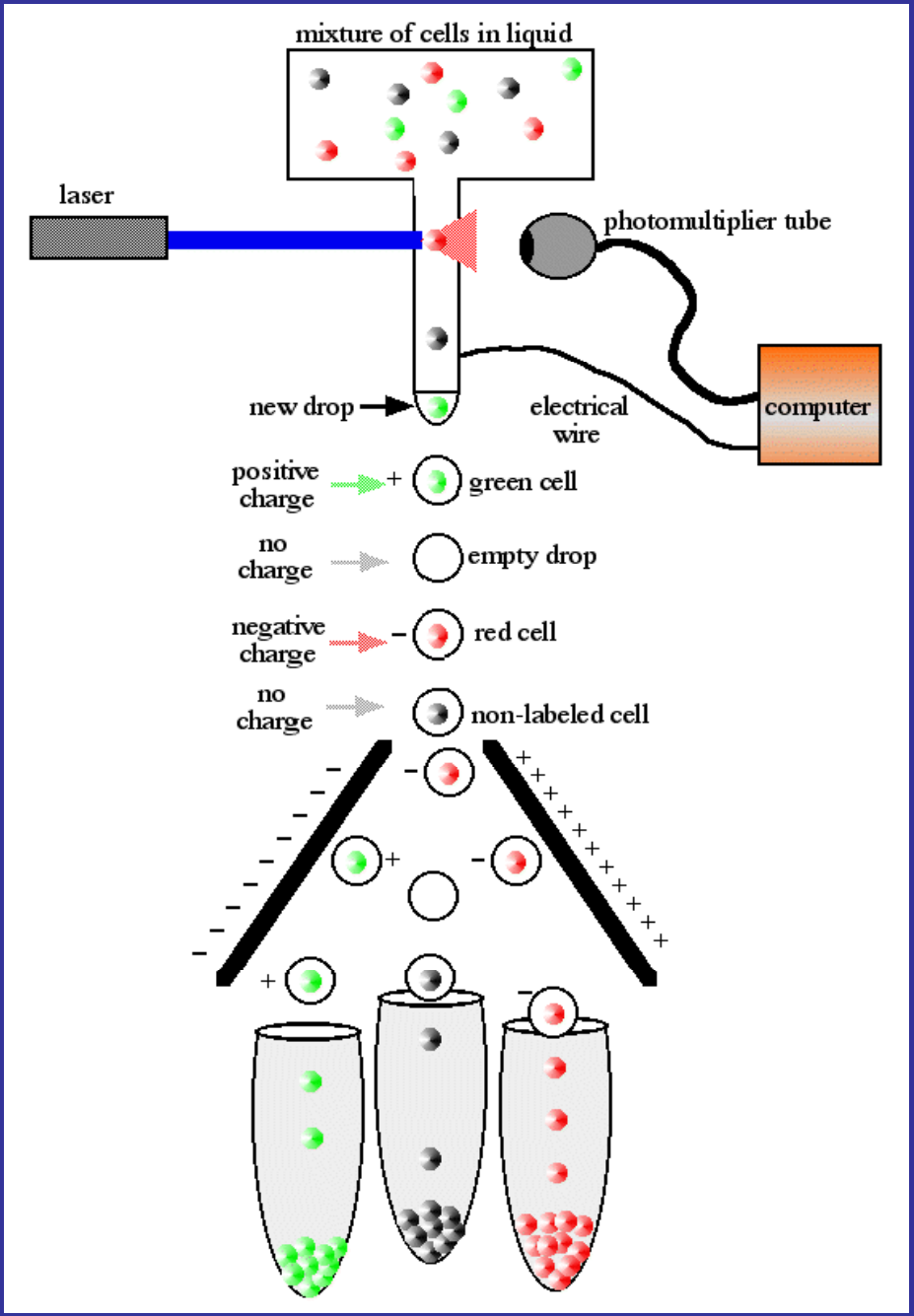
- The voltage pulse height, width, and area are determined by the particle's size, speed, and fluorescence intensity.
- The pulse parameters are then acquired and analyzed in real-time by a computer.

Flow Cytometer Instrumentation
Graphical Summary



In Addition...

- Some flow cytometers can sort cells into pre-determined subpopulations.
- An electrostatic charge is used to deflect a drop containing a fluorescently-labeled cell into one of three collection vessels.



www.bio.davidson.edu

Fluorescence-activated Cell Sorters

BD FACSVantage

BD FACSAria

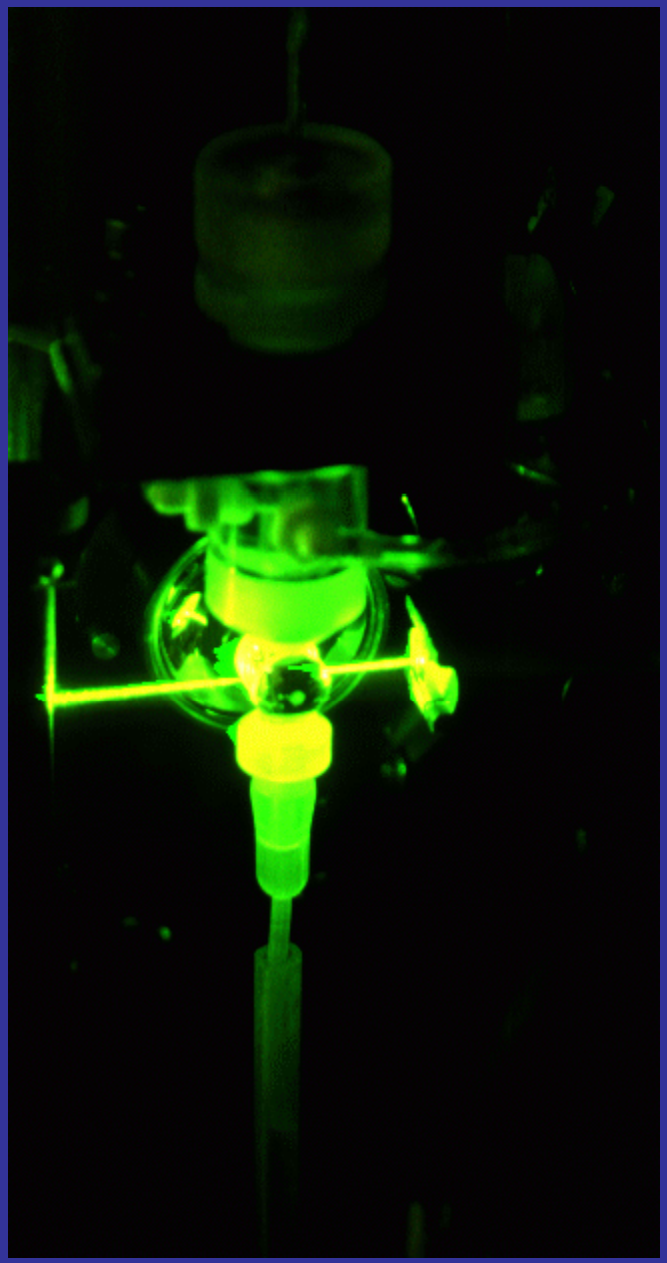
Benchtop Flow Cytometers

BD FACSCalibur

BD LSR II

Flow Cell

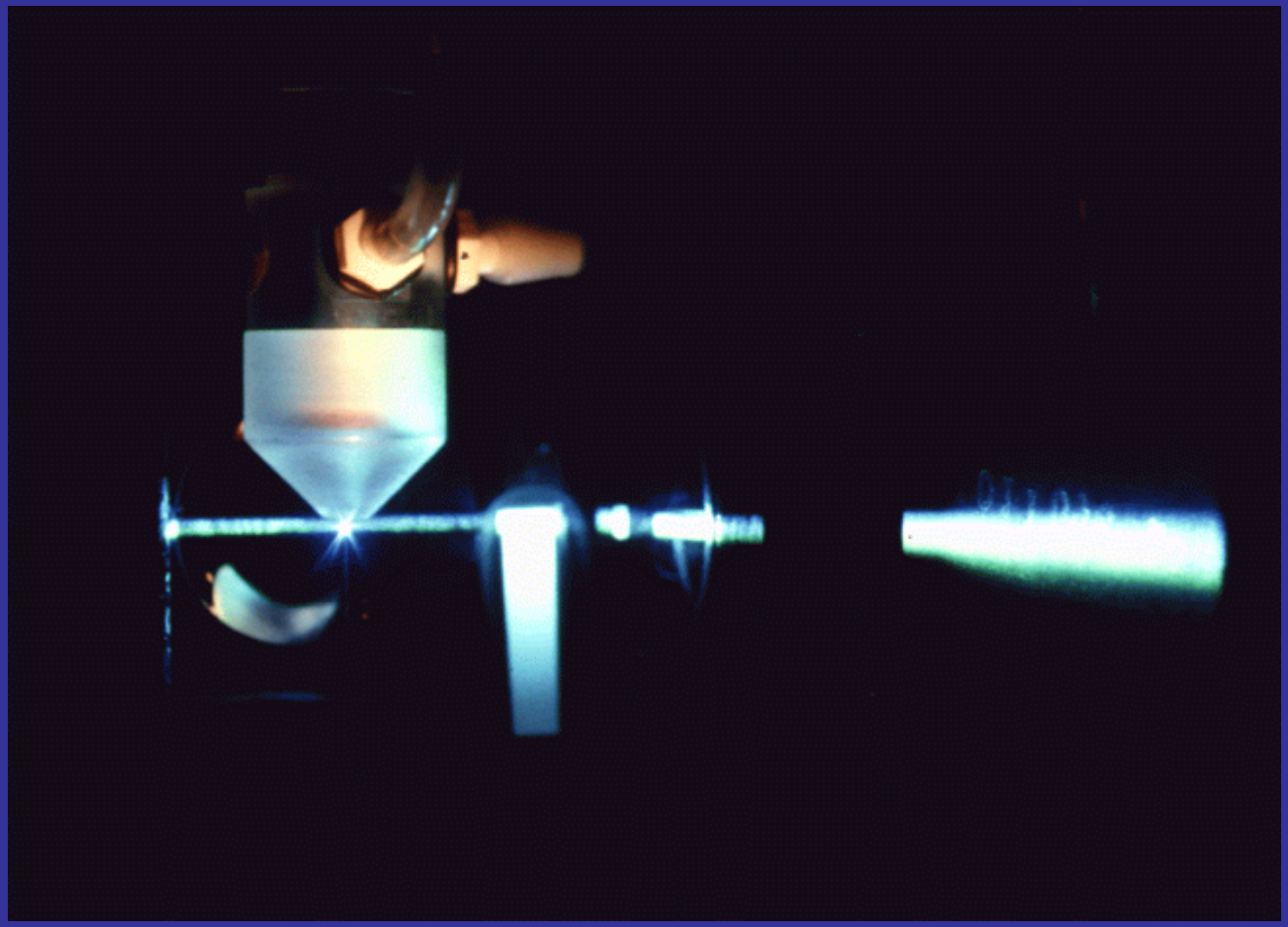
- The flow cell is the flow chamber where the laser beam interrogates the particles passing within the sheath fluid.
- This is typically a closed system.



www.cyto.purdue.edu

Nozzle Tip

- The flow chamber inside a cell sorter is stream-in-air, rather than laminar flow.



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- This is typically an open system.

Differentiating Among Cell Types

- In the early days of flow cytometry, different cell types were identified based only on their light scattering characteristics.
- Even though thousands of cells could be rapidly detected, flow cytometry offered little more than what could be achieved by cell counters and microscopy.
- The introduction of fluorochromes into flow cytometry converted this otherwise limited method of cell detection into a powerful tool for the rapid differentiation of cells.

Fluorochrome-conjugated Antibodies

- Initially, fluorescent dyes commonly employed in microscopy were used to stain whole cells.

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- However, dye uptake by cells was unreliable and led to problems with data reproducibility.

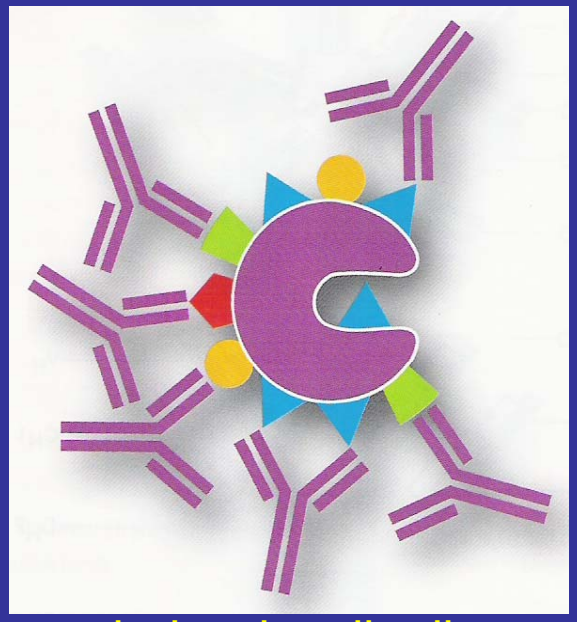
- Subsequently, antibodies were covalently bound to fluorochromes as a means of specifically and reliably labeling cells.

Basic Immunology

- Antibodies (immunoglobulins) are proteins used by the immune system to neutralize foreign invaders.
- They recognize, through specific binding, molecules called antigens.
- Antigens are ubiquitous in nature. They are found in the body, as well as in foreign invaders.
- The antibody-antigen interaction has many uses in the laboratory, including the specific identification of cells.

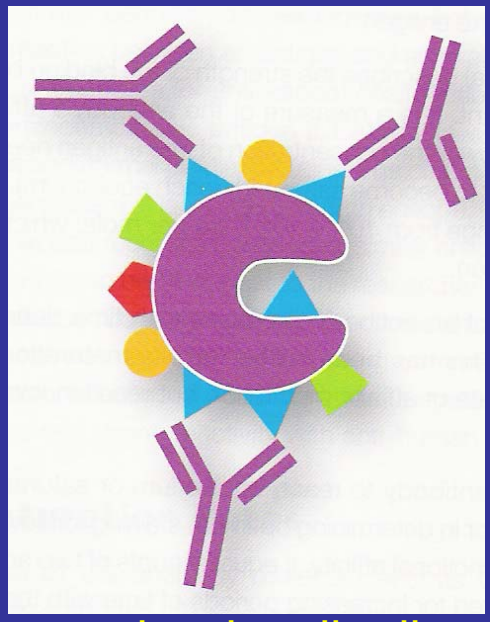
Polyclonal vs. Monoclonal Antibodies

- Polyclonal antibodies bind to multiple aspects of the same antigen. Their heterogeneity causes problems with standardization when used in flow cytometry.



polyclonal antibodies

- Homogeneous monoclonal antibodies bind to only one aspect of an antigen and will reproducibly label cells.



monoclonal antibodies

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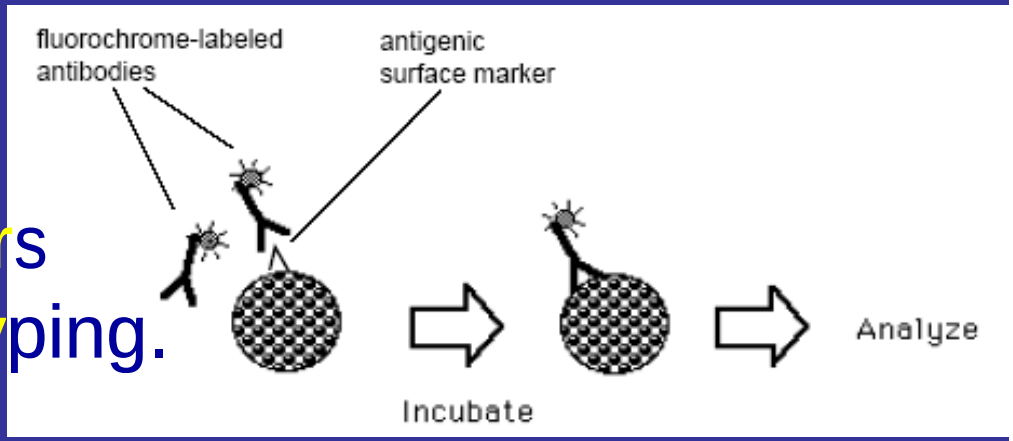
Immunophenotyping

- Monoclonal antibodies are used to recognize specific antigens on the surface of cells.
- These cell-surface markers characterize different cell types.
- Fluorochrome-tagged monoclonal antibodies brightly label cells for detection by the flow cytometer.
- Identification of cells using cell-surface markers is called immunophenotyping.



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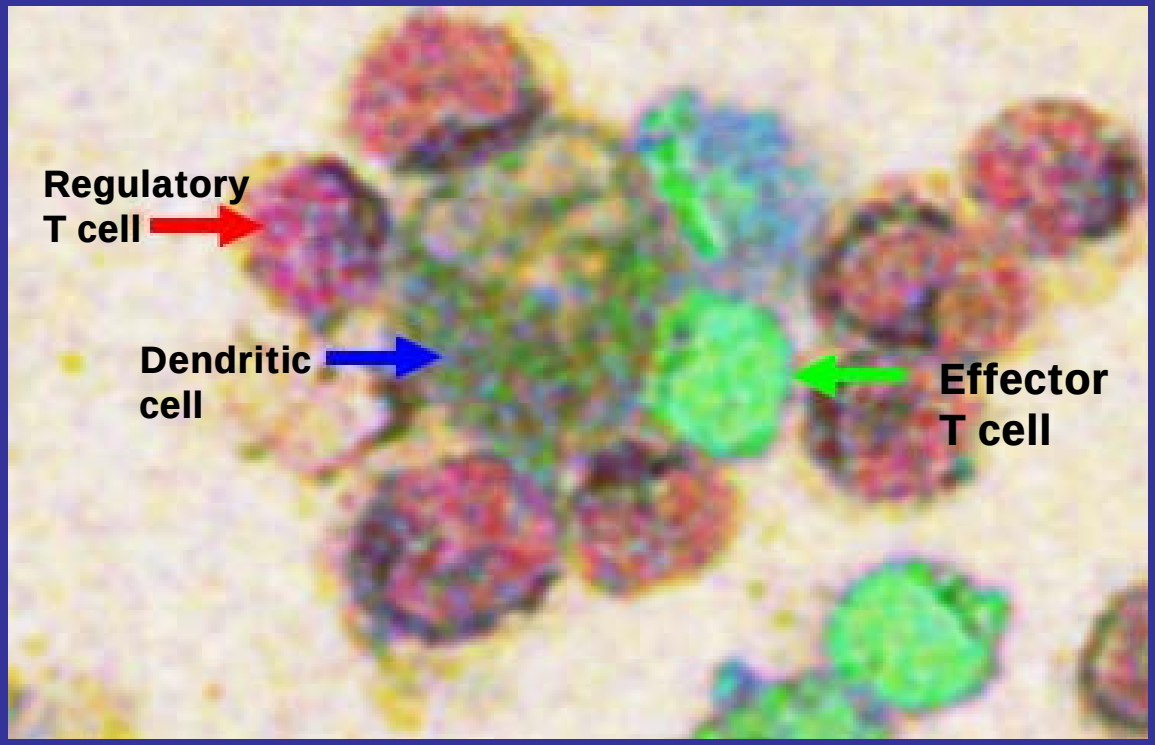
Immunophenotyping

- In immunology, the use of fluorochrome-tagged monoclonal antibodies resulted in the discovery of phenotypically diverse T cell subsets.



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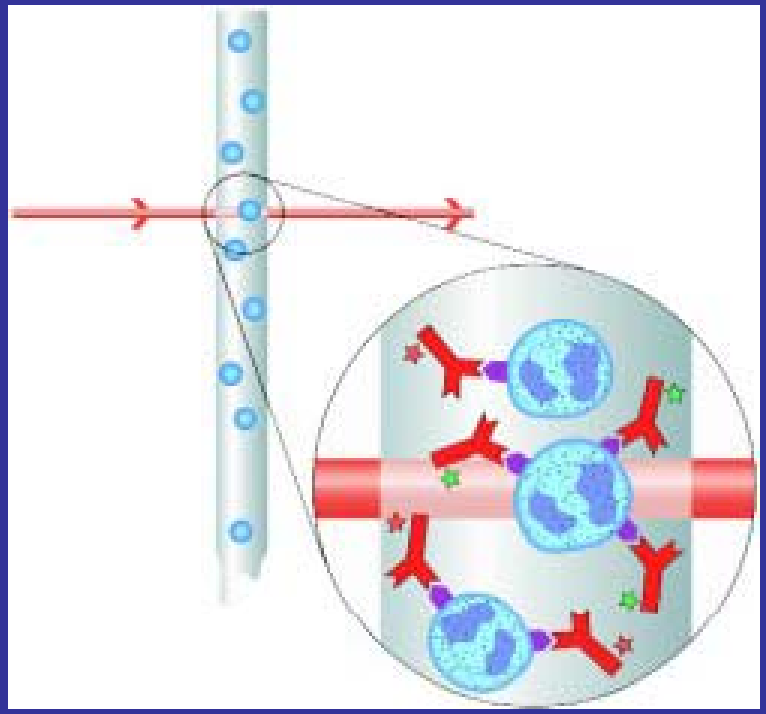
- This revolutionary observation made flow cytometry the preferred research tool of modern immunology.

Immunophenotyping

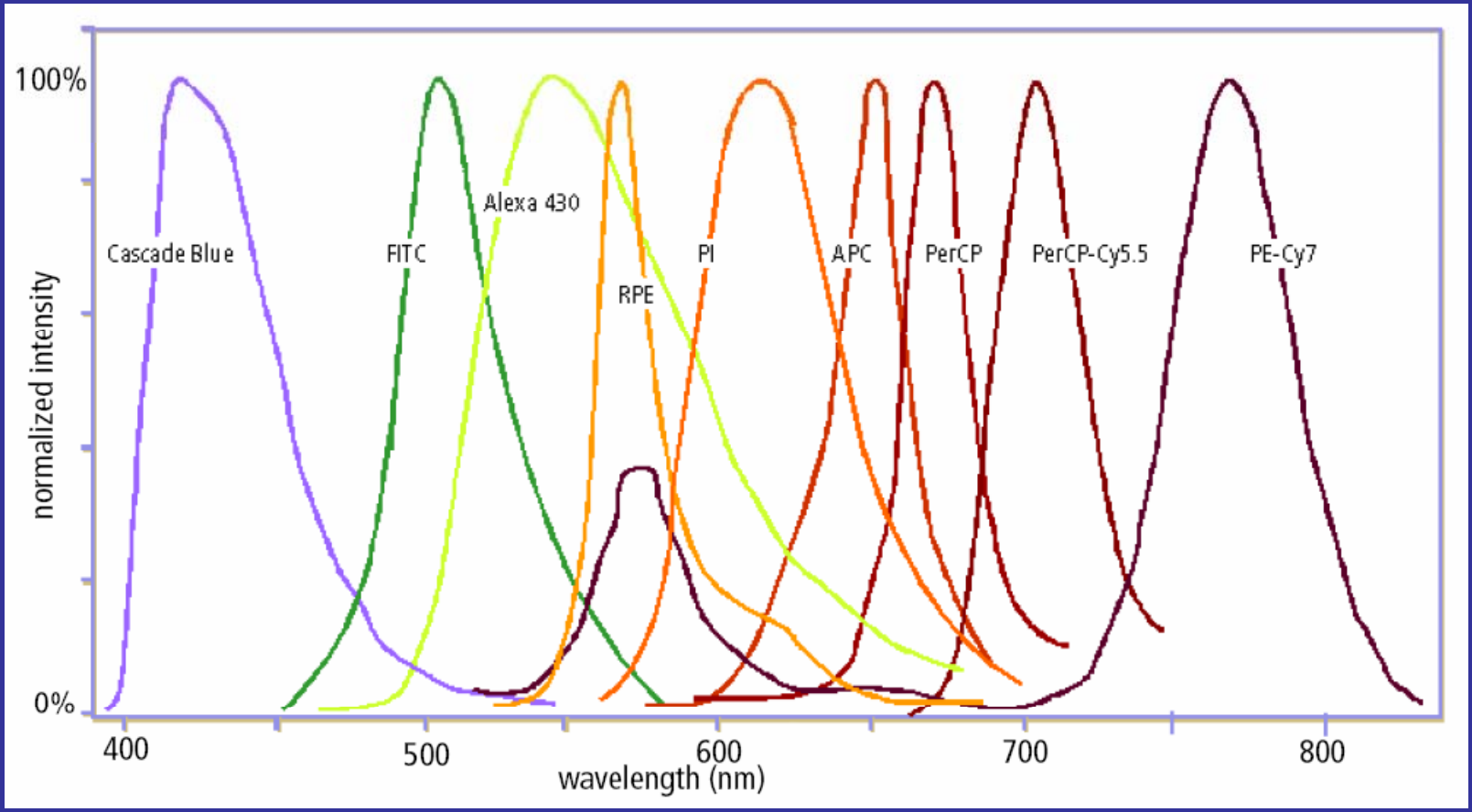
- Many cell surface features (as well as some internal characteristics) can be simultaneously assessed by employing different combinations of fluorochromes.
- Several uniquely colored fluorochromes are available to conduct such multicolor (multiparameter) experiments.



www.serotec.com

Immunophenotyping

- However, many fluorochromes possess overlapping emission wavelengths.



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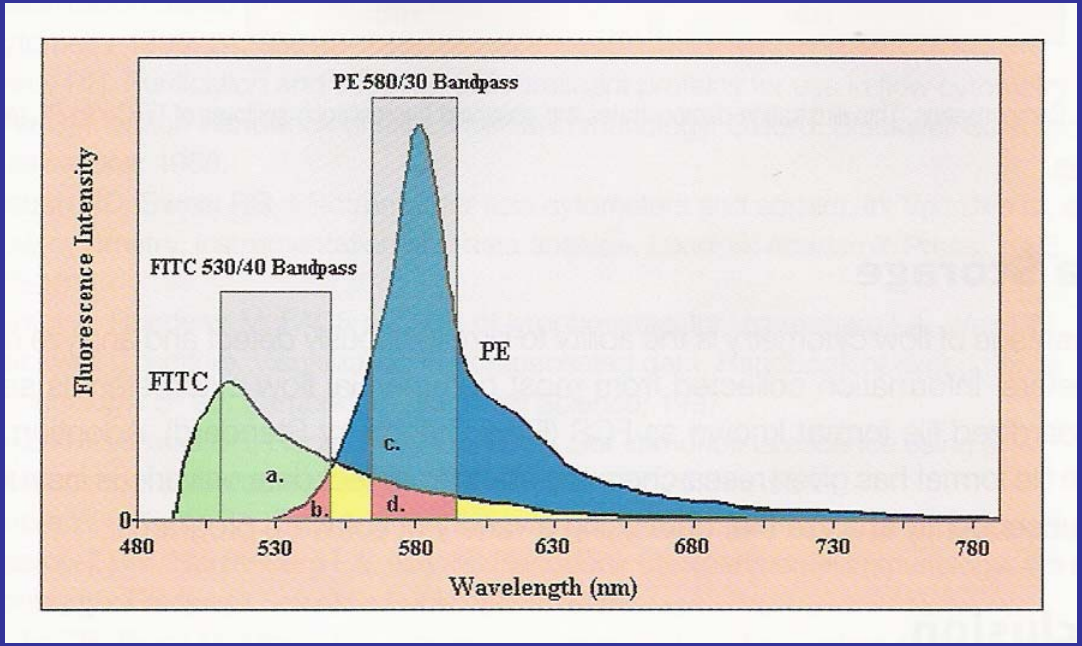
Compensation

- When the wavelengths of two fluorochromes overlap, the observed fluorescent signal detected by the flow cytometer may not be the actual signal displayed by the cell.
- In other words, the cell appears to possess a surface marker or phenotype that it does not actually have.



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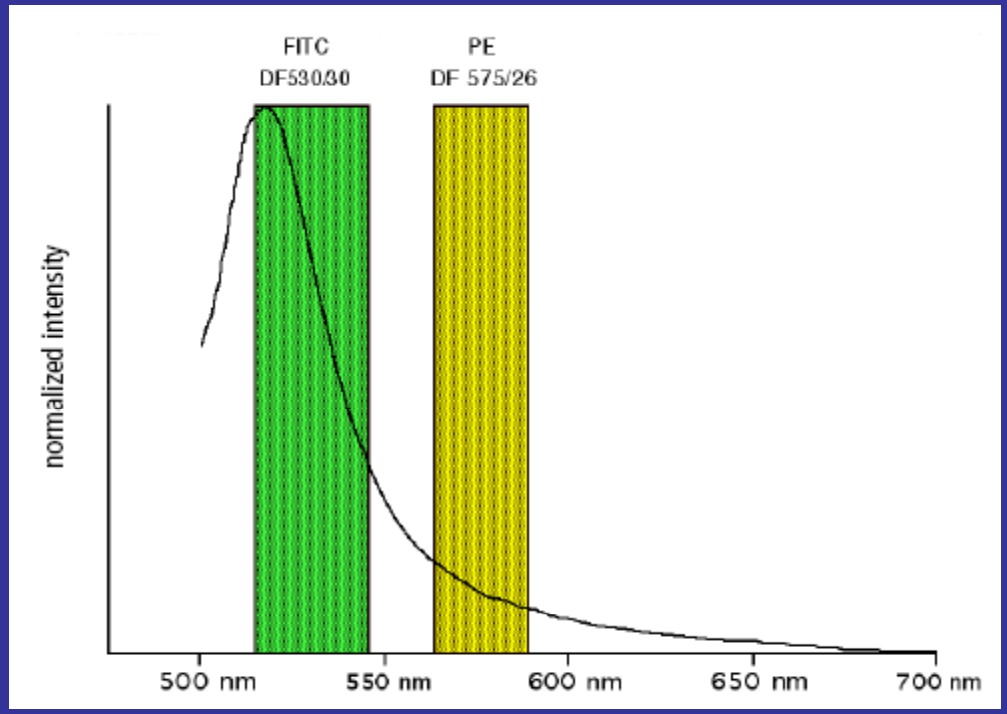
Compensation

- This fluorescence interference can be corrected for by adjusting the measurement parameters of the flow cytometer (either manually or automatically).
- This correction is termed compensation.
- In addition, this problem can be avoided by carefully selecting fluorochromes that do not overlap.



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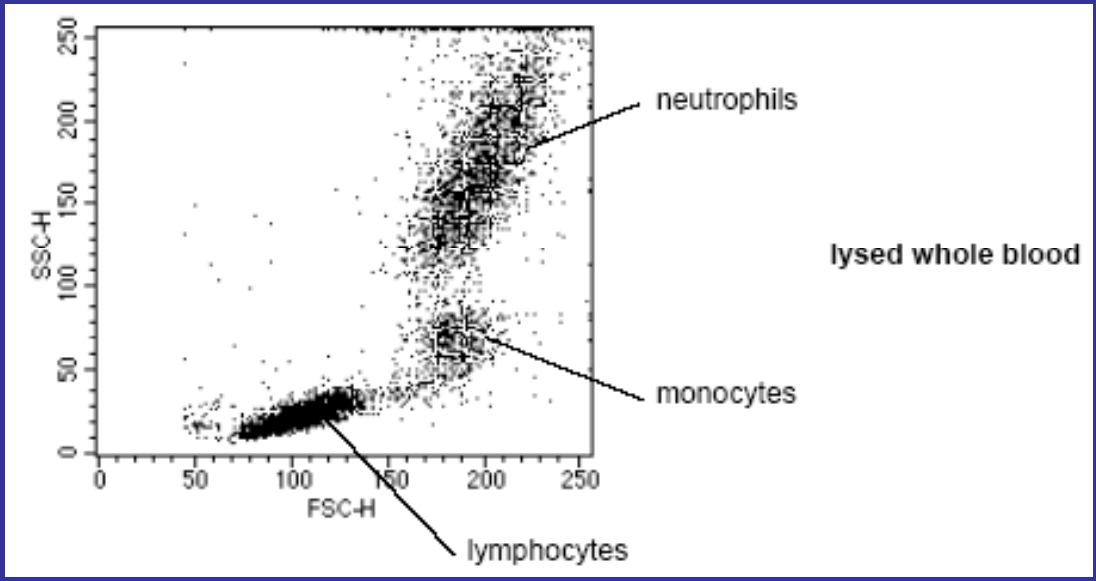
Data Analysis

- Flow cytometry is utilized both in the clinical lab and the research lab.
- Standardization has resulted in data that is reproducible across laboratories.
- Accurate data representation is key to this reproducibility.
- This is a 2D dot plot; a commonly used method of data representation.



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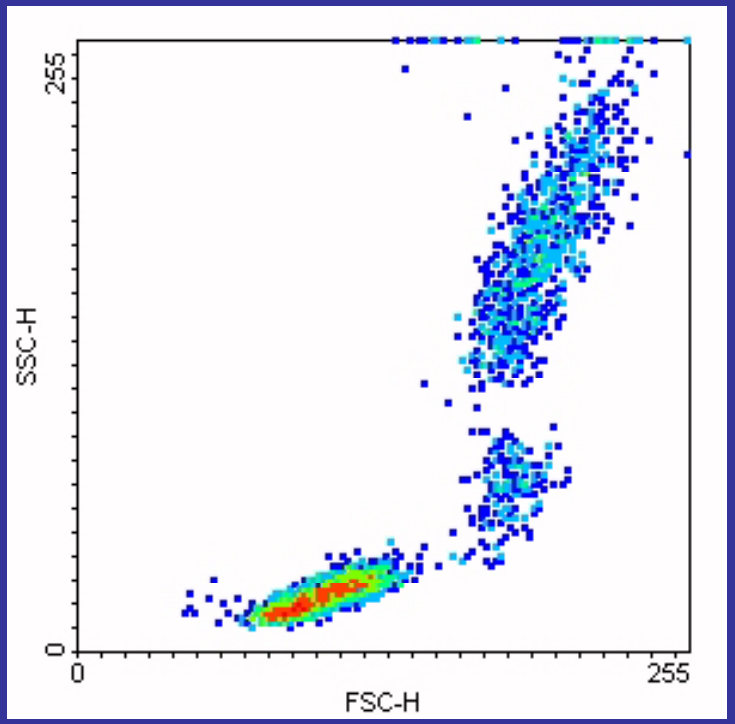
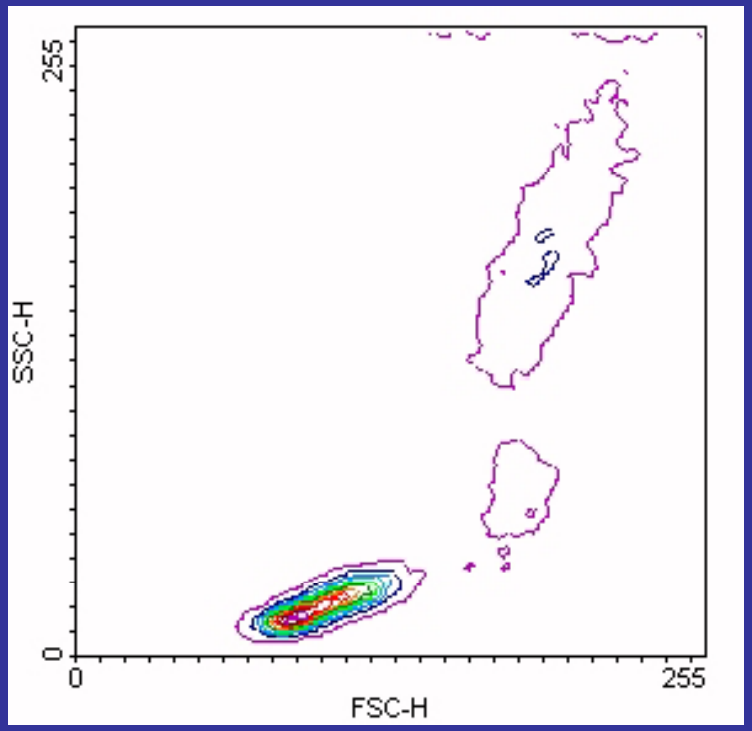
Data Analysis

- Flow cytometry computer software can generate data in the form of density plots and contour plots.
- These graphical representations can sometimes be misleading.





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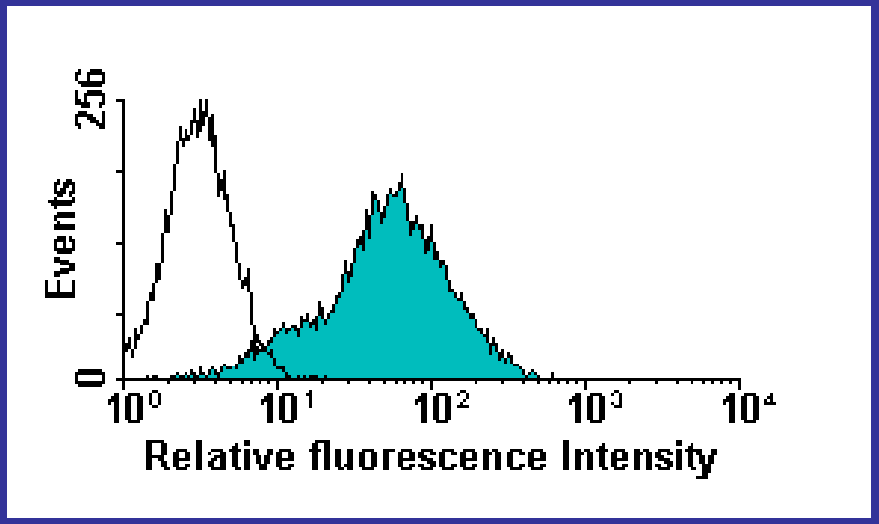
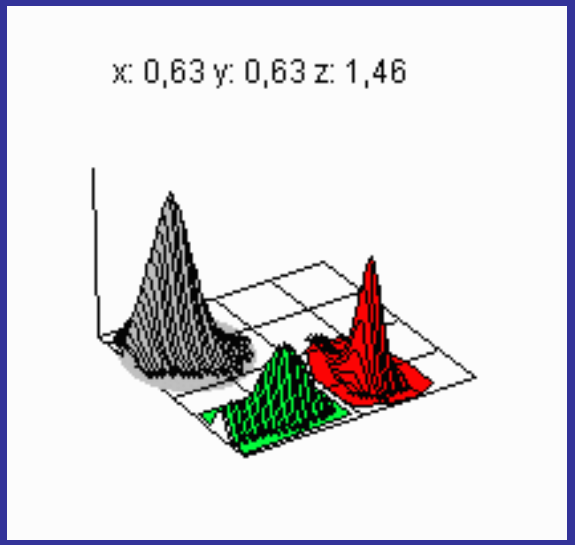
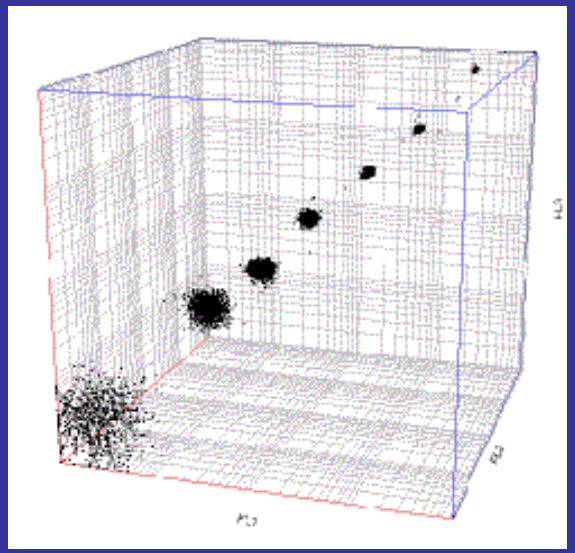
Data Analysis

- Histograms are a common and reliable method used to present flow data for analysis.



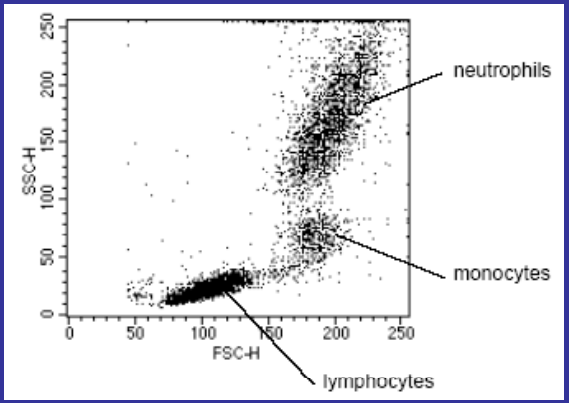


- However, these graphs require ⟷ advanced software and are more visual than useful.

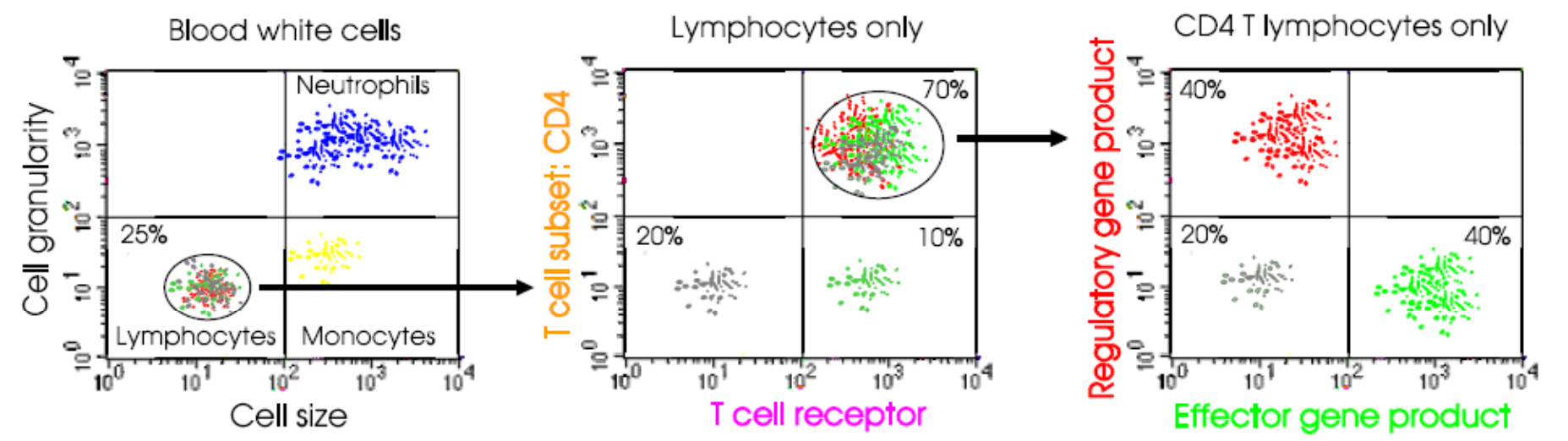


Gating

- To optimize the analysis of multiparameter experiments, gating is performed to isolate cell subpopulations of interest.



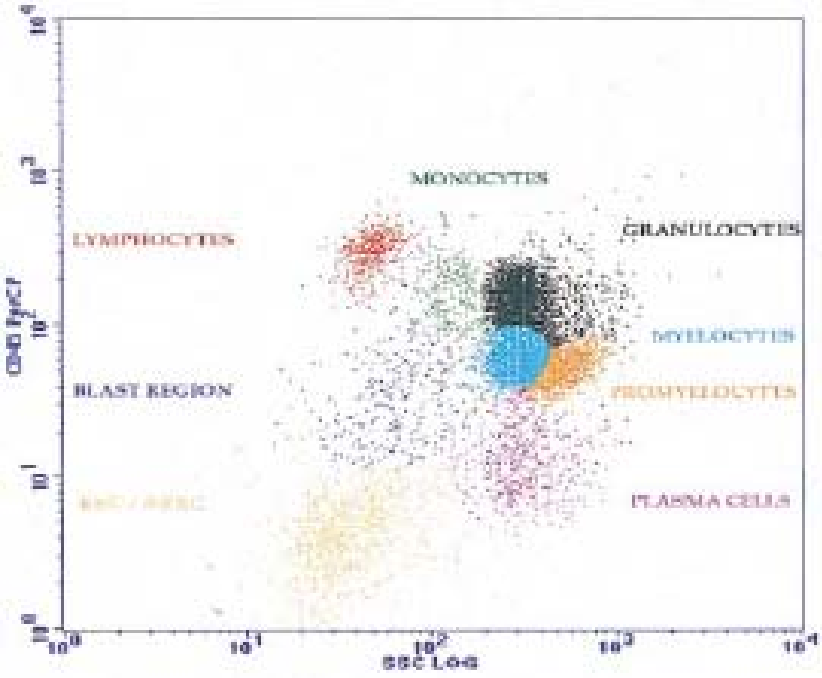
- This step often eliminates the need to physically sort cells for further analysis.



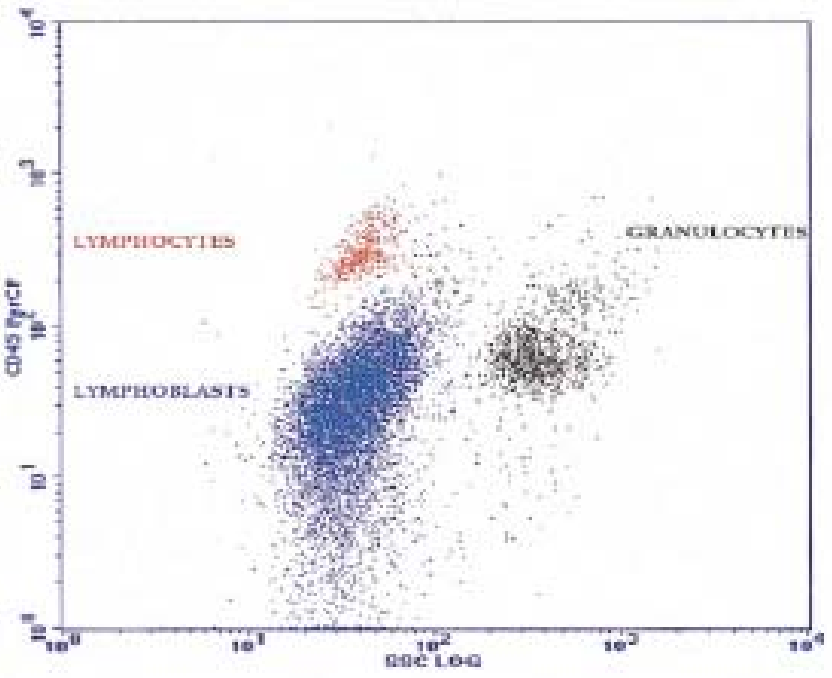
Applications - Clinical

- Bone marrow cells are evaluated based on SSC and CD45 expression to diagnose acute lymphoblastic leukemia.

normal patient



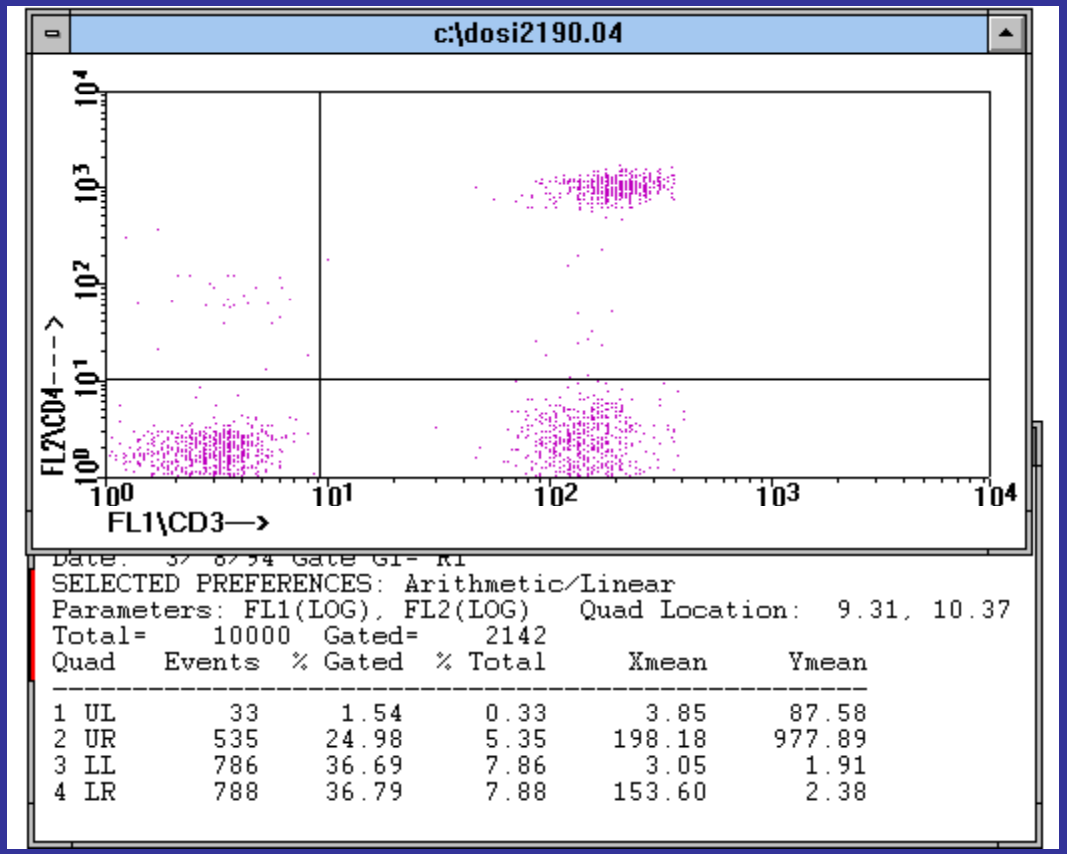
patient with ALL



Jennings, C. & Foon, K.(1997). *Blood, 90*(8), 2863-2892.

Applications - Clinical

- $CD4^+$ T cell counts are used to monitor the progression of AIDS in HIV-infected patients.



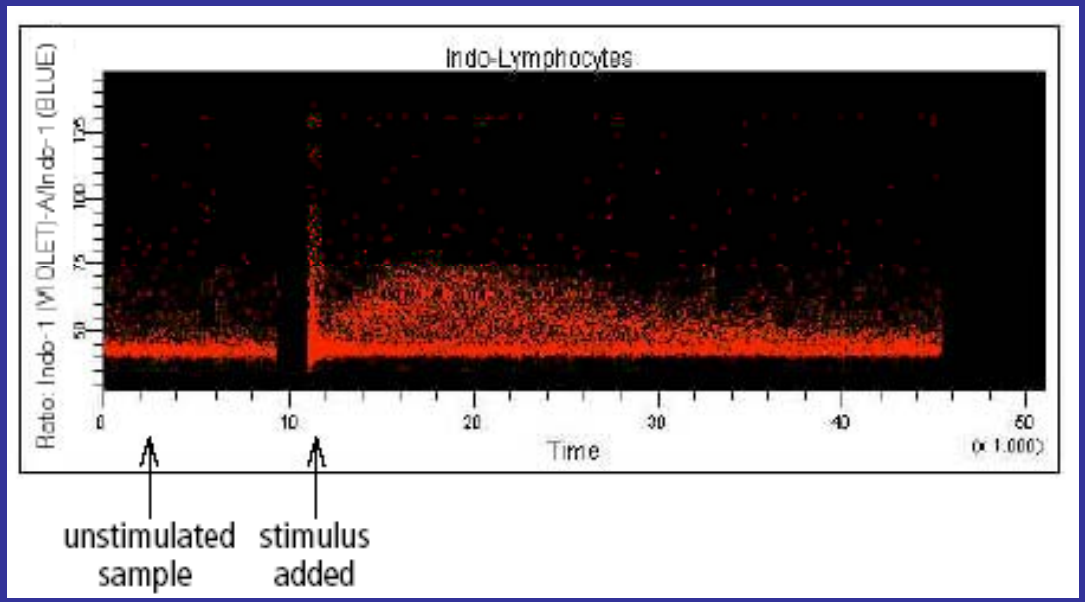
LABORATORY REPORT

Client Name: Smith, Bob		**ID Number:**	692271440
Date Drawn: 5/21/04		**Date of Report:**	5/29/04
Account Number: 12687			
Test Name:	**Result**	**Reference Range**	
HIV 1 RNA PCR	265 (High)	<50 Copies/mL	
Absolute CD4+ Cells	102 (Low)	490–1740 per CMM	

www.medic.med.uth.tmc.edu

Applications - Research

- A kinetics assay, such as Ca^{2+} mobilization, can be performed using a fluorochrome, indo-1, that binds to calcium ions.
- Cells are loaded with indo-1 and then stimulated to mobilize Ca^{2+}.
- The UV laser excites the indo-1 and a fluorescent pulse is observed over time.



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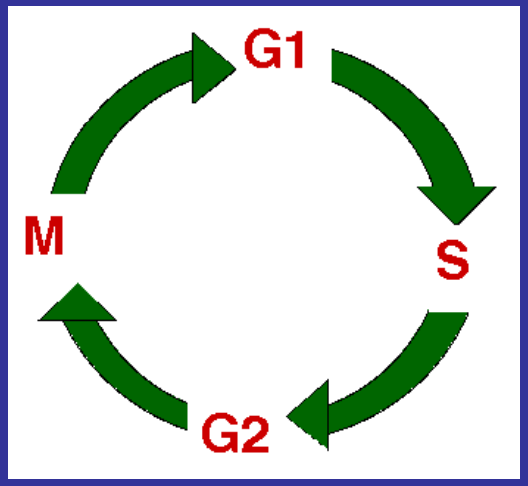
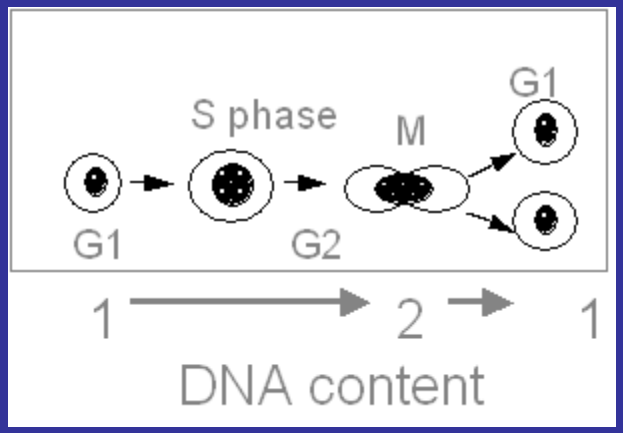
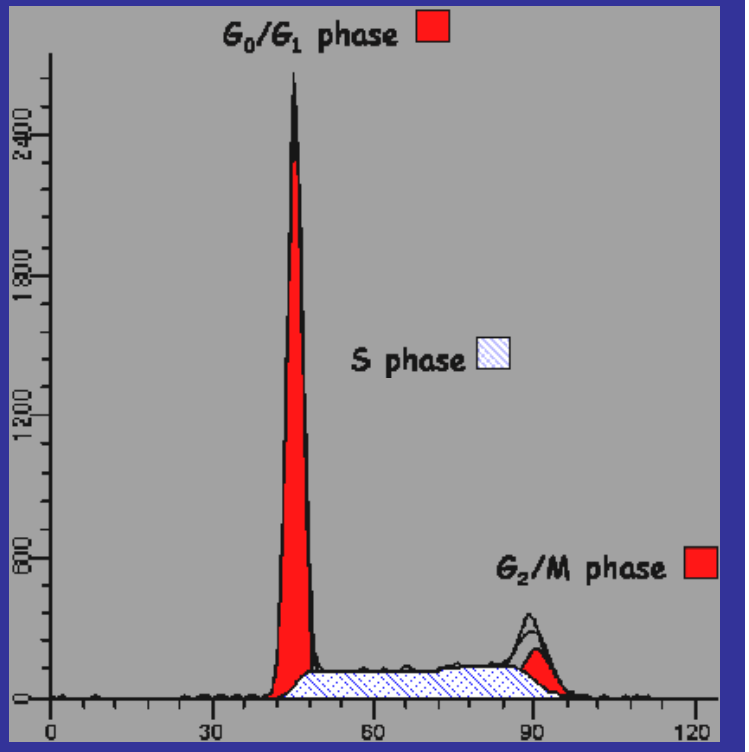
Applications - Research

- Several fluorochromes (DAPI, propidium iodide, 7-AAD, etc.) bind directly to DNA and are used to estimate the amount of DNA present in a cell.
- The amount of DNA in a cell indicates whether or not it has entered the cell cycle.







www.xenbase.org

Summary

- Flow cytometers measure cells based on their size, internal complexity, and fluorescence.
- Qualitative and quantitative analyses of cell populations have clinical and research applications.
- Successful experimental design depends on an understanding of flow cytometer instrumentation and basic immunological principles.